Caspase Activation in High-Pressure–Induced Apoptosis of Murine Erythroleukemia Cells

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Abstract: Murine erythroleukemia cells were subjected to physicochemical stresses such as high pressure (60–110 MPa), heating (42–45°C), and ultraviolet (UV) irradiation (5–25 kJ/cm²). After exposure to these stresses, the cells were cultured at 37°C and atmospheric pressure. The number of the cells treated at 100 MPa, 45°C, or 20 kJ/cm² remained constant or decreased at an early stage of culture. The nuclear morphology, agarose gel electrophoresis, and flow cytometry of these cells showed that they undergo apoptosis. The activity of caspase-3 was observed in

cells subjected to each stress. In particular, caspase-3 was most readily activated by high pressure under our conditions. The caspase-3 activity and apoptosis exhibited a similar pressure dependence. It is important that both caspase-3 activation and apoptosis induced by high pressure were significantly suppressed by a caspase-3 inhibitor. These results suggest that high-pressure-induced apoptosis is also characterized by the activation of caspase-3, as seen with heat- and UV-induced apoptosis. [Japanese Journal of Physiology, 51, 193–199, 2001]

Key words: apoptosis, caspase-3, high pressure, heat, UV.

The cell death is classified into apoptosis and necrosis [1, 2]. Apoptosis or programmed cell death plays an important role in morphogenesis and maintenance of tissue homeostasis [3]. Furthermore, apoptosis is an evolutionary conserved form of cell death after exposure to external stresses such as ultraviolet (UV) irradiation [4, 5] and antitumor agents [6, 7]. Apoptotic cell death is characterized by a series of morphological and biochemical changes, including cell shrinkage, microvilli loss, membrane potential loss, chromatin condensation, and nucleosomal DNA fragmentation [8]. On the other hand, the necrotic cell death reveals mitochondria swelling, cell rupture, and release of proinflammatory agents [9]. It is well known that many caspase family members are involved in these apoptotic processes [2, 3, 10]. However, recent research has shown that there is caspase-3–independent pathway in apoptosis [11, 12].

Previously, we demonstrated that when murine erythroleukemia (MEL) cells were exposed to a pressure

of 80 MPa and then cultured at atmospheric pressure, the cells in S phase were sensitive to high pressure and were arrested in G2 phase [13]. At higher pressures, the decline in cell density was observed. In the present work, we have examined the properties of cell death induced by high pressure. These results have been compared with the properties of cell death by heating or UV irradiation.

MATERIALS AND METHODS

Chemicals. Ethidium bromide, proteinase K, dithiothreitol (DTT), and Triton X-100 were obtained from Wako Chemicals. Propidium iodide (PI) and ribonuclease A (RNase A) were obtained from Sigma. Acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC) and acetyl-Asp-Glu-Val-Asp-aldehyde (Ac-DEVD-CHO) were from Peptide Institute, Inc. Nonidet P-40 (NP-40) was from Nacalai Tesque. All other chemicals were of reagent grade.

Received on June 28, 2000; accepted on January 12, 2001

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Abbreviations: Ac-DEVD-CHO, acetyl-Asp-Glu-Val-Asp-aldehyde; Ac-DEVD-AMC, acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin; DTT, dithiothreitol; PBS, phosphate buffered saline (136 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.4); PI, propidium iodide; RNase A, ribonuclease A; MEL, murine erythroleukemia; NP-40, Nonidet P-40; UV, ultraviolet.

Cell culture. MEL cells (cell line 745A) were maintained in RPMI-1640 medium containing 10% fetal calf serum, streptomycin (0.1 mg/ml), and penicillin G (100 U/ml) at 37°C in a CO₂ (5%) incubator.

High-pressure, heat, and UV treatments. Cells ($0.5\text{--}1.0 \times 10^6$ cells/ml) in the medium were subjected to various pressures (0.1–110 MPa) for 30 min at 37°C [13, 14] or to various temperatures (37 to 45°C) for 30 min or 1 h at atmospheric pressure. For UV irradiation, the cells ($0.5\text{--}1.0 \times 10^6$ cells/ml) were suspended in phosphate-buffered saline (PBS; 136 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.4) and exposed to various doses (0 to 25 kJ/cm²) at 365 nm, using a UV generator (model FLX-20M, Vilber Lourmat, France), under atmospheric pressure. After irradiation, the cells were resuspended in the medium. These stress-exposed cells were cultured for 4 d at 37°C and atmospheric pressure. The cell number was calculated by using a hemocytometer and an Olympus light microscope (model BHC).

Morphological changes of nuclei. MEL cells exposed to physicochemical stresses were incubated in 75 mM KCl for 5 min at room temperature and fixed with methanol/acetic acid (3:1, v/v) for 20 min at room temperature. The samples were mounted onto glass slides and stained with Giemsa. The nuclear morphology was examined by using a light microscope.

Cell cycle analysis by flow cytometry. The suspensions of stress-exposed cells ($1.0\text{--}2.0 \times 10^6$ cells) were centrifuged for 5 min at $250 \times g$ and 4°C. The cells were washed twice with PBS, then fixed with 70% ethanol overnight at –20°C. The samples were washed with PBS and treated with RNase A (100 µg/ml) in PBS for 20 min at 37°C. After treatment, the cells were washed once in PBS and stained with PI (50 µg/ml) for 10 min at room temperature. Flow cytometric analysis was performed by using a FACScan (Becton-Dickinson) or a EPICS XL System II (Coulter).

Agarose gel electrophoresis of DNA. The suspensions of stress-exposed cells ($1.0\text{--}2.0 \times 10^6$ cells) were centrifuged for 5 min at $250 \times g$ and 4°C, and the cells were washed twice with PBS. The cells in 50 µl of PBS were mixed with 50 µl of 100 mM Tris-HCl, 10 mM EDTA, pH 8.0 (Tris buffer) containing 0.8% SDS and proteinase K (100 µg/ml), then incubated overnight at 50°C. The proteins were excluded with phenol/chloroform (1:1, v/v). The DNA in aqueous phase was precipitated by the addition of ice-cold ethanol. After washing with 70% ethanol, the precipitate was dissolved in Tris buffer containing

RNase A (100 µg/ml), then incubated for 1 h at 37°C. After reprecipitation with ethanol, DNA was dissolved in Tris buffer containing 0.8% SDS, then mixed with sample buffer (10 mM EDTA, 0.05% SDS, 5% glycerol, and 0.025% bromophenol blue). Agarose gel (1.5%) electrophoresis was performed in electrophoresis buffer (89 mM Tris-HCl, 89 mM boric acid, 2.5 mM EDTA, pH 8.4). After electrophoresis, gels were stained with ethidium bromide (10 µg/ml).

Measurement of caspase activity. For the measurement of caspase-3 activity, MEL cells were preincubated for 2 h at 37°C in the presence or absence of 100 µM Ac-DEVD-CHO, then exposed to stresses such as high pressure (60–100 MPa, 37°C, 30 min), heating (45°C, 30 min), or UV irradiation (20 kJ/cm², 365 nm). These stress-exposed cells were cultured at atmospheric pressure in the presence or absence of 100 µM Ac-DEVD-CHO for 1.5 h for high-pressure- and heat-treated samples or for 24 h for UV-irradiated ones. After the culture, the cells were washed twice in chilled PBS, then treated with aqueous solution containing 1% Triton X-100 and 1% NP-40. The lysate was incubated for 30 min at 4°C and centrifuged for 5 min at $17,000 \times g$ and 4°C. The supernatant and a substrate (Ac-DEVD-AMC, final concentration 50 µM) in 100 mM HEPES-KOH, 5 mM DTT, 10% NP-40, 10% sucrose, pH 7.4 were mixed, and the mixture (0.5 ml) was incubated for 1 h at 37°C. After incubation, 2 ml of sodium acetate (1 M, pH 4.2) was added to the reaction mixture. Released AMC was measured at 380 nm excitation and 460 nm emission. Aliquots of high-pressure-treated cells (60, 80, 100 MPa) were used to measure apoptosis using a flow cytometry. To examine the effect of caspase-3 inhibitor on high-pressure-induced apoptosis, MEL cells were preincubated for 1 h at 37°C in the presence of Ac-DEVD-CHO (200 and 500 µM), exposed to a pressure of 100 MPa for 30 min at 37°C in the absence of the inhibitor, then cultured in the presence of Ac-DEVD-CHO (200 and 500 µM) for 1.5 h. For the measurement of apoptosis using a flow cytometry, these cells were washed twice with PBS, fixed in 70% ethanol, and stained with PI as mentioned above.

Statistics. All values are expressed as mean $\pm$ SE, unless otherwise noted. Where appropriate, a Student's *t*-test for paired data was used to assess the significance of difference.

RESULTS

Effects of high pressure, heating, and UV irradiation on proliferation of MEL cells

MEL cells were exposed to high pressure (60, 80,

100, or 110 MPa), then cultured at atmospheric pressure. The cell growth curves were similar to those previously reported (Fig. 1A) [13]. Namely, the cell proliferation was significantly suppressed at 80 MPa. In 100 MPa-treated cells, the cell density decreased after 1 d of culture, but then increased. Upon exposure to a pressure of 110 MPa, most cells were fragmented before culture. In this case, the cell density remained at a low level (below 10^4 cells/ml) during the culture. In the present work, therefore, we have focused on the decrease of cell density in 100 MPa-treated cells. Furthermore, we examined the effects of other physicochemical stresses such as heating (37 to 45°C) and UV irradiation (5 to 25 kJ/cm²) on cell growth. The cell density of heated cells (Fig. 1B) or UV-irradiated ones (Fig. 1C) was significantly decreased above 44°C or 20 kJ/cm², compared with that of 37°C-treated cells or UV-unexposed ones, respectively. In the case of 45°C, no such destruction of cells as seen with 110 MPa was observed immediately after heating, though the cell density greatly decreased after 1 d of culture. So we chose 100 MPa, 45°C, and 20 kJ/cm² as a pressure, temperature, or UV irradiation for cell damage, respectively.

Apoptosis in high-pressure-, heat-, and UV-treated MEL cells

To characterize the cell damages, the morphological changes of nuclei in 100 MPa-, 45°C-, or 20 kJ/cm²-treated MEL cells were examined by light microscopy (Fig. 2). In each case, the nuclear condensation and fragmentation were observed. These results suggest a possibility of apoptotic cell death [8]. So the cell cycle and DNA state of these samples were analyzed by flow cytometry and agarose gel electrophoresis, respectively. Figure 3 shows typical DNA histograms of exponentially growing cells and stress-exposed ones. The cells in G1 and G2/M phases bear the DNA contents corresponding to 2 and 4 N, respectively. The DNA histogram of 100 MPa-treated cells, which were not cultured after decompression, was similar to that of untreated cells. However, the cells with DNA contents below 2 N appeared during the culture. Such cells correspond to apoptotic ones. Similarly, apoptotic cells appeared with the culture of 45°C-treated cells. On the other hand, UV-irradiated cells also showed a signal below 2 N. However, the appearance of these apoptotic cells was late compared with the case of 100 MPa-, or 45°C-treated cells.

The DNA ladder, which results from DNA fragmentation by endonucleases, is widely used to characterize the apoptotic cells. So the DNA fragmentation in the cells exposed to the physicochemical stresses

Fig. 1. Effects of physicochemical stresses on proliferation of MEL cells. (A) MEL cells were exposed to various pressures for 30 min at 37°C, then cultured at atmospheric pressure. (B) The cells were subjected to various temperatures for 1 h and then cultured at 37°C. (C) The cells were exposed to various doses of UV at 365 nm, then cultured. The cell density was calculated by using a hemocytometer. Values are the mean $\pm$ SE of at least three independent experiments. The densities of stress-treated cells (80 MPa, 44°C, 20 kJ/cm²) at indicated times were decreased significantly, compared with those not stress treated ($p < 0.05$).

was examined by agarose gel electrophoresis (Fig. 4). DNA fragmentation in high-pressure (100 MPa)-, heat (45°C)-, or UV-treated (20 kJ/cm²) MEL cells appeared after 1.5, 1.5, or 24 h, respectively.

Fig. 2. Morphological changes of nuclei in MEL cells exposed to physico-chemical stresses. MEL cells exposed to (B) 100 MPa at 37°C for 30 min, (C) 45°C for 1 h, or (D) 20 kJ/cm² at 365 nm were cultured for 1, 1.5, or 24 h, respectively. These cells were fixed and stained with Giemsa. The cells were observed by using a light microscope. A: Stress-unexposed cells (control). Arrows indicate apoptotic cells.

Fig. 3. DNA histograms of stress-exposed MEL cells. MEL cells exposed to pressure (100 MPa, 37°C, 30 min), heat (45°C for 1 h), or UV (20 kJ/cm²) were cultured for 0 to 6 h (until 24 h for UV). The cells were fixed and stained with PI for flow cytometric analysis.

Caspase activity in high-pressure-, heat-, and UV-treated MEL cells

To characterize apoptosis induced by high pressure,

Fig. 4. DNA ladder in MEL cells exposed to physico-chemical stresses. MEL cells exposed to pressure (100 MPa, 37°C, 30 min), heat (45°C, 1 h), or UV (20 kJ/cm²) were cultured for 0 to 6 h (until 24 h for UV). DNA isolated from these cells was analyzed by agarose gel electrophoresis. C, DNA from stress-unexposed cells.

heating, or UV irradiation, the activity of caspase-3 in 100 MPa-, 45°C-, or 20 kJ/cm²-treated MEL cells was examined by using a fluorescence substrate, Ac-DEVD-AMC. Judging from fluorescence intensity in the presence or absence of a caspase-3 inhibitor Ac-DEVD-CHO [15, 16], it appears that caspase-3 was activated in each case (Fig. 5). In particular, high pressure, compared with heating and UV irradiation, most effectively activated caspase-3 under our conditions. Regarding the cells with DNA contents below 2N as apoptosis, we examined a relation between apoptosis and caspase-3 activity in high-pressure-treated MEL

cells (Fig. 6). When the cells were exposed to various pressures (60, 80, and 100 MPa), the caspase-3 activity and the population of apoptotic cells both increased significantly above 80 MPa. It is interesting that the population of apoptotic cells considerably decreased upon the addition of 200 μ M Ac-DEVD-CHO (Fig. 7). Similar results were also obtained at a higher concentration (500 μ M) of the caspase-3 inhibitor (data not shown).

Fig. 5. Caspase-3 activation in MEL cells exposed to physicochemical stresses. MEL cells were preincubated for 2 h at 37°C in the presence (+) or absence (-) of 100 μ M Ac-DEVD-CHO and subjected to 100 MPa, 45°C, or 20 kJ/cm². Pressure- or heat-treated cells were cultured in the presence or absence of inhibitor for 1.5 h at 37°C, whereas UV-treated cells were similarly cultured for 24 h. These cells were washed and lysed to measure caspase activity, using 50 μ M Ac-DEVD-AMC. Values are the mean $\pm$ SE of three independent experiments. Caspase-3 activity induced by each stress was significantly suppressed by Ac-DEVD-CHO ($p < 0.01$). C, untreated cells.

Fig. 6. Apoptosis and caspase-3 activation as a function of high pressure. MEL cells were exposed to various pressures (60, 80, and 100 MPa) for 30 min at 37°C, then cultured for 1.5 h at atmospheric pressure (0.1 MPa). These cells were used to measure apoptosis by flow cytometry and caspase-3 activity. The percentage of the cells with DNA contents below 2N is shown as %Apoptosis. Values are the mean $\pm$ SE of three independent experiments. * $p < 0.01$ vs. apoptosis at 0.1 MPa; ** $p < 0.05$ vs. caspase-3 activity at 0.1 MPa.

Fig. 7. Inhibition of apoptosis by caspase-3 inhibitor in 100 MPa-treated MEL cells. MEL cells were preincubated for 1 h at 37°C in the absence (B) or presence (C) of 200 μ M Ac-DEVD-CHO and exposed to 100 MPa at 37°C for 30 min. After decompression, the cells were cultured for 1.5 h in the absence (B) or presence (C) of Ac-DEVD-CHO (200 μ M). Pressure-untreated cells (A) and pressure-treated ones (B, C) were stained with PI for flow cytometric analysis. The cells with DNA contents below 2N are presented by the percentage. Values are the mean $\pm$ SE of three independent experiments. * $p < 0.05$ vs. (B).

about 130 MPa. Thus high pressures of 80 to 100 MPa seem to be more suitable for an investigation of the cell cycle.

Several lines of evidence have demonstrated that a pressure of 100 MPa, as well as heating and UV irradiation, can also induce apoptosis in MEL cells. High-pressure (100 MPa)-induced apoptosis was reported in human lymphoblasts [18]. However, the mechanism of this apoptosis is not yet known. So it is useful to compare high-pressure-induced apoptosis with UV- or heat-induced apoptosis. UV irradiation, which is widely used to induce apoptosis [4, 5], produces DNA damages such as cyclobutane pyrimidine dimers, whereas γ radiation produces single- or double-strand breaks [4]. Excision repair systems may be activated in UV-irradiated cells. If such DNA damages cannot be repaired, the cells would undergo apoptosis. On the other hand, are there DNA damages in 100 MPa- or 45°C-treated cells? In general, high pressure stabilizes DNA double strands [19], whereas it dissociates oligomeric proteins such as actin filaments [20]. It is well known that the DNA double helix is readily denatured at high temperature ($\sim 100^\circ\text{C}$). However, the denatured single strands form native double helix under subdenaturing conditions ($\sim 65^\circ\text{C}$). Heating (42°C) of human monoblastoid cell line U937 cells induces heat shock proteins (*hsps*) such as *hsps* 27, 70, and 90 [7]. These proteins increase the resistance to apoptosis-inducing agents such as actinomycin D and etoposide [7]. These facts suggest that the compression at 100 MPa and the heating at 45°C may affect the stability of proteins rather than DNA under our conditions. Thus the cause of high-pressure- or heat-induced apoptosis seems different from that of UV-induced apoptosis.

Recent studies on apoptosis demonstrate that caspase-3 plays a crucial role in the execution of programmed cell death [2, 3]. Caspase-3 activates deoxyribonuclease that degrades DNA during apoptosis [21]. On the other hand, caspase-3 itself is activated by caspase-9, which is activated by cytochrome c [3]. Thus the release of cytochrome c into the cytosol from mitochondria serves as a trigger to activate caspases [22]. Therefore it is of interest to examine whether cytochrome c is released from mitochondria into the cytosol in high-pressure-induced apoptosis. From an assay using fluorescence substrate, we have found that caspase-3 activation depends on high pressure, and caspase-3 is greatly activated in 100 MPa-treated MEL cells. Furthermore, caspase-3 activity and apoptosis induced by high pressure are both significantly suppressed by Ac-DEVD-CHO. Further studies are necessary whether lack of complete sup-

pression of apoptosis by Ac-DEVD-CHO reflects the low membrane permeability of the inhibitor or the contributions of other factors besides caspase-3. On the basis of these data, we conclude that caspase-3 plays an important role in high-pressure-induced apoptosis.

As described above, apoptosis is induced in eukaryotic cells by high pressure, as well as by heating and UV irradiation. On the other hand, there are prokaryotes capable of growing under extreme conditions such as a pressure of 100 MPa [23] and the boiling temperature of water [24]. Other bacteria can also inhabit more extreme environments such as salt brines. Thus prokaryotic cells have become adapted to extreme environments because of their simpler cell organizations compared with eukaryotic cells. In contrast, eukaryotic cells, which acquired more complicated cellular structures and functions under atmospheric pressure, have integrated the mechanism of cell death to eliminate unnecessary cells or damaged ones during the evolution. Thus it seems likely that the application of an unphysiological pressure to eukaryotic cells also triggers cell suicide. The present work indicates that MEL cells exposed to a pressure of 100 MPa under our conditions undergo apoptosis. Further work is necessary to clarify how caspase-3 is activated in high-pressure-induced apoptosis.

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